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MOLECULAR INSIGHTS INTO THE PATHOGENESIS OF B-CELL AND MYELOID MALIGNANCIES: THERAPEUTIC IMPLICATIONS

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Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-129-9

MOLECULAR INSIGHTS INTO THE PATHOGENESIS OF B-CELL AND MYELOID MALIGNANCIES: THERAPEUTIC IMPLICATIONS

THESIS FOR DOCTORAL DEGREE (Ph.D)

The thesis will be defended in public at CMM lecture hall, 9:00 am, 2021-03-26

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Dedicated to my beloved family

POPULAR SCIENCE

Not all diseases get cured completely. Hematological malignancies are cancers derived from blood/bone marrow or lymphatic tissues and are divided into many subtypes. How these malignancies are formed is incompletely understood. For many patients diagnosed with these cancers, the current treatment needs great improvement to gain better clinical efficacy. Thus, it is essential to explore the mechanisms underlying these hematological malignancies, thereby developing new treatment strategies.

Hodgkin lymphoma (HL) is a malignant disease derived from B cells. HL development is thought to be related to many factors, including age, gender difference, familial and genetic risk, environmental factors, etc. We described monozygotic triplets, carrying the same DNA profiles. Two of them developed HL, while the other remained HL free. We found that these two HL triplets had certain similarity in some markers located on top of the DNA sequence of their hematopoietic cells. The finding might be helpful for better understandings of the development of HL.

Myeloproliferative neoplasms (MPNs) are a group of myeloid disease derived from the bone marrow. The treatment of MPNs is mostly not curative. Janus kinase 2 (commonly called JAK2) is an enzyme that is involved in some cell signaling pathways and the development of cancers. A JAK2 inhibitor LY2784544 could affect the activity of an important signal pathway in normal human life as well as the formation of cancers. In a large number of MPN patients, there is a gene mutation occurring in the JAK2 gene called $JAK2^{V617F}$. Increased JAK2 activity leads to the accumulation of progenitor and mature blood cells. There is an enzyme in our body called telomerase which is important in cell life-span, and cancer development. We found that the drug GRN163L targeting telomerase together with a JAK2 inhibitor might be potentially used as a novel treatment strategy for MPNs.

The enzyme 5-LOX is generally thought to be related to the metabolism of lipids. It has been reported to be related to some B cell-related diseases. We found that this enzyme is correlated with the migration and adherence of mantle cell lymphoma (MCL). Inhibiting the migration and adhesion of MCL cells to secondary lymphatic tissues can block the protection malignant cells get by crosstalk with the microenvironment. Compounds that inhibit 5-LOX may be useful in the treatment of certain B-cell malignancies.

ABSTRACT

Hematological malignancies originate from hematopoietic cells in the bone marrow or the lymphatic system. In this thesis, I study the molecular pathogenesis of Hodgkin lymphoma (HL), mantle cell lymphoma (MCL), and myeloproliferative neoplasms (MPNs), and explore the underlying therapeutic implication in these diseases.

In **paper I,** we profiled the DNA methylation signature in blood cells from monozygotic triplets, two of which had developed HL. Isolated CD34+ hematopoietic cells, marginal zone-like and naïve B-cells, and switched memory B-cells were compared for the difference in global DNA methylation between the triplets with the one without HL. Shared differences were found in naïve B-cells and marginal zone-like B-cells. One region in chromosome 18 was found to be hyper-methylated in the naïve B-cells, while 21 genomic regions on 10 chromosomes were hyper-methylated in marginal-zone like B-cells derived from the HL triplets. These findings may indicate a role of aberrant DNA methylation in HL pathogenesis.

In **paper II**, we probed new therapeutic strategies for MPNs. The activating $JAK2^{V617}$ mutation drives MPN development. Treatment with JAK2 inhibitors frequently leads to therapeutic failure, and the underlying mechanism is elusive. We observed that the JAK2 inhibitor LY2784544 caused the transient decrease in viability and proliferation of the $JAK2^{V617}$-bearing cell line HEL, but induced the accumulation of leukemic stem cell (CD34+) cells. The stem cell factor KLF4 was highly expressed upon LY2784544 treatment, whereas silencing of KLF4 attenuated the accumulation of LY2784544-induced accumulation of CD34+ cells. A similar effect was also identified in cells treated with the telomerase inhibitor GRN163L after LY2784544 incubation. Thus, targeting KLF4 combined with a JAK2 inhibitor, or combination of telomerase inhibitor and JAK2 inhibitors may potentially be used as a novel treatment strategy for MPN patients.

In **paper III**, we investigated the role of 5-lipoxygenase (5-LOX) in MCL and evaluated the effect of inhibition of 5-LOX as a therapeutic strategy. Human B lymphocytes express 5-LOX and 5-LOX-activating protein (FLAP), and can convert arachidonic acid to leukotriene B_4. MCL cells express large amounts of 5-LOX, but the function and mechanism of activation of 5-LOX in B lymphocytes are unclear. We demonstrated that the intrinsic 5-LOX pathway in the MCL cell line JeKo-1 has an essential role in the migration and adhesion of these cells. 5-LOX and FLAP inhibitors reduced the migration induced by the chemoattractant CXCL12. The knockout of the 5-LOX gene also decreased the migration of JeKo-1 cells. Furthermore, the above inhibitors inhibited adherence of JeKo-1 cells to stromal cells. The inhibitors were as potent as the Bruton tyrosine kinase inhibitor ibrutinib, a therapeutic agent in MCL treatment, to inhibit migration and adherence of MCL cells. Our results suggest that inhibition of the 5-LOX pathway may be an important strategy in the treatment of certain B-cell malignancies.

Taken together, the findings presented in this thesis contribute to better understandings of the molecular pathogenesis of HL, MCL, and MPNs, and are implicated in the rational development of novel therapeutic strategies for these malignancies.

LIST OF SCIENTIFIC PAPERS

I. **Chuanyou Xia**, Thale Kristin Olsen, A. Ali Zirakzadeh, Radwa Almamoun, Louise K Sjöholm, Jenny Dahlström, Jan Sjöberg, Hans-Erik Claesson, John Inge Johnsen, Ola Winqvist, Dawei Xu, Tomas J Ekström, Magnus Björkholm, Klas Stråat
Hodgkin Lymphoma Monozygotic Triplets Reveal Divergences in DNA Methylation Signatures.
Front Oncol. 2020;10:598872. doi: 10.3389/fonc.2020.598872. eCollection 2020

II. Jenny Dahlström, **Chuanyou Xia**, Xiangling Xing, Xiaotian Yuan, Magnus Björkholm, Dawei Xu
JAK2 inhibition in JAK2^{V617F}-bearing leukemia cells enriches CD34+ leukemic stem cells that are abolished by the telomerase inhibitor GRN163L
Biochem Biophys Res Commun. 2020;527(2):425-431. doi: 10.1016/j.bbrc.2020.04.058. Epub 2020 Apr 22.

III. **Chuanyou Xia**, Laia Sadeghi, Klas Stråat, Magali Merrien, Anthony P. Wright, Birgitta Sander, Dawei Xu, Anders Österborg, Magnus Björkholm, Hans-Erik Claesson
Intrinsic 5-lipoxygenase activity regulates migration and adhesion of mantle B-cell lymphoma cells.
Manuscript.

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LIST OF ABBREVIATIONS

5-HETE	5-Hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxide eicosatetraenoic acid
5-LOX	5-lipoxygenase
APC	Allophycocyanin
BCA	Bicinchoninic acid assay
BEA	Bioinformatic and Expression Analysis Core Facility
BTK	Bruton´s tyrosine kinase
cHL	Classical Hodgkin lymphoma
CYSLTR1	Cysteinyl leukotriene receptor 1
DC	Dendritic cells
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ET	Essential thrombocythemia
FDA	Food and Drug Administration
FLAP	5-lipoxygenase-activating protein
HL	Hodgkin lymphoma
HRS	Hodgkin and Reed/Sternberg cells
JAK2	Janus kinase 2
JH	Janus homology domain
LDHL	Lymphocyte depleted classical HL
LOX	Lipoxygenase
LRHL	Lymphocyte rich classical HL
LT	Leukotriene
LTs	Leukotrienes
MCHL	Mixed cellularity classical HL
MCL	Mantle cell lymphoma
MiRNA	Micro Ribonucleic acid
MKL1	Megakaryoblastic leukemia 1
MPNs	Myeloproliferative neoplasms
NHL	Non-Hodgkin lymphoma

NSHL	Nodular sclerosis classical HL
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PMF	Primary myelofibrosis
PV	Polycythemia vera
PVDF	Polyvinylidene difluoride
q-PCR	Quantative Real-time polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SFM	Serum-free medium
SNPs	Single nucleotide polymorphisms
TERC	Telomerase RNA template
TERT	Telomerase reverse transcriptase
TRAP	Telomeric repeat amplification protocol
WB	Western blot

1 INTRODUCTION

Hematological malignancies originate from hematopoietic cells in the bone marrow or the lymphatic system. Although there has been a lot of improvement in the clarification of these diseases, current treatments are not always satisfying. It is therefore of importance to further gain insights into the pathogenesis of these malignancies for rational development of novel treatments. We performed this study on the molecular pathogenesis of Hodgkin lymphoma (HL), mantle cell lymphoma (MCL), and myeloproliferative neoplasms (MPNs), exploring the underlying therapeutic implication in these diseases.

Our group previously reported a male monozygotic triplet case, two of these triplets were diagnosed with HL, while the other one is free of HL. We found that there is a deletion in the first intron of the megakaryoblastic leukemia 1 (MKL1) gene on chromosome 22 in all of these three triplets (1). To further explore whether epigenetic factors affect the development of HL, we examined the Deoxyribonucleic acid (DNA) methylation profiles of four cell subpopulations derived from peripheral blood mononuclear cells (PBMCs) of the triplets, and our findings could be of importance in the understanding of HL pathogenesis.

A poor prognosis of patients with MPNs is sometimes related to resistance-treatment with JAK 2 inhibitors. $JAK2^{V617F}$ is a gain-of-function mutation that is present in the vast majority of MPNs, and JAK2 inhibitors have moderate efficacy in the treatment of MPNs. Telomerase activation is very common in many types of cancers (2), and a telomerase inhibitor, GRN163L, was reported to show promising effects in the treatment of MPNs and some other hematological diseases (3-5). Here we report our findings in a human erythroleukemia cell line HEL which carries the $JAK2^{V617F}$ mutation, aiming to find out whether the telomerase inhibitor and JAK2 inhibition shows synergistic effect in the treatment of MPNs.

MCL is derived from B lymphocytes, these malignant cells have a relative high-expression of 5-lipoxygenase (5-LOX) (6-8). 5-LOX belongs to the lipoxygenase family and the enzyme converts fatty acid to leukotrienes (LTs) which are important mediators of allergic and immune reactions. Here we have studied the role of 5-LOX pathway in the migration and adhesion in MCL cell line JeKo-1.

2 LITERATURE REVIEW

2.1 HODGKIN LYMPHOMA

Hodgkin lymphoma (HL) is a type of lymphoma, formerly called Hodgkin´s disease which originates from the lymphatic system. It was named after Thomas Hodgkin who was the first pathologist describing this disease (9). HL tumors have a unique feature in that the tumor contains only 1-2% malignant cells (Hodgkin and Reed/Sternberg cells, HRS) which are believed to originate from a post germinal centre B-cell. The rest of the tumor microenvironment mainly harbours inflammatory cells of the immune system, stromal cells, macrophages, eosinophils, mast cells and plasma cells (10).

2.1.1 Epidemiology

In 2018, about 80,000 new cases were estimated to contribute to the newly diagnosed cancer patients in the world and 26,000 patients died from HL (11). The estimated age-standardized incidence was approximately 2.4 cases per 100,000 persons per year, and the number of deaths was about 0.33 person per 100,000 persons per year in Europe.

HL is divided into two major groups according to the appearance of tumor cells and the composition of inflammatory background: classical Hodgkin lymphoma and Nodular lymphocyte predominant HL. Classical Hodgkin lymphoma (cHL) can then be subdivided into four subgroups, Nodular sclerosis classical HL (NSHL), Mixed cellularity classical HL (MCHL), Lymphocyte rich classical HL (LRHL), Lymphocyte depleted classical HL (LDHL). Nodular sclerosis subtype is the most common cHL which accounts for about 70%-80% of all HL cases, mixed cellularity cHL accounts for about 15% (12), and the other two types of HL are rare. HL has a bimodal age-incidence pattern, it has a high incidence peak for children and young adults, the second incidence peak appears at an older age.

2.1.2 HL and families

Numerous studies have shown that genetic factors are involved in the HL pathogenesis. Goldin et al (13) collected and analyzed the data from first-degree relatives of patients with HL and the first-degree relatives of control groups from Sweden and Denmark. The results suggested that, although the relative risks were not the same, the risk of relatives of HL was higher compared with control groups in both Sweden and Denmark, and the incidence risk among siblings was higher among the first-degree relatives. The difference was more obvious in males than females (14). Besides, the risk was almost double in same-sex siblings than opposite-sex siblings. Mack et al (15) identified 432 sets of twins affected by HL, the monozygotic twins of

the patients were found to have greater incidence risk; and 10 pairs of the monozygotic twins showed concordance, while no correlation was shown in dizygotic twins. A Swedish national cohort study in which 3.57 million individuals were involved showed that the children or young adults who had HL familial history in a sibling or parent had about 8.83 and 7.19 higher risk to develop HL, respectively (16).

2.1.3 HL and epigenetics

Epigenetics explains how the phenotype can be changed without change of DNA sequences. The cellular and phenotype change may be caused by external and environmental factors, as well as the alteration happening in normal development. DNA methylation and histone modification are the two major mechanisms that cause epigenetic alterations. The methylation of CpG sites in the promoter region mediates the silence of gene transcription.

On the phenotypic level there can be quite pronounced differences between monozygotic twins, and many studies have shown that epigenetic differences may contribute to these differences (17). Fraga et al (18) found that monozygotic twins have different epigenetic signatures, which provides a new insight for scientists in studying the role of epigenetic factors in phenotype differences. Wang´s group (19) compared the DNA methylation differences in twins, they found a higher DNA methylation level at the *TCF3* locus in HL survivors than their unaffected twins, however, no difference was found in the healthy-twin groups.

Hyper-methylation was mostly found in the promoter region of tumor suppressor genes. Ying et al (20) found that aberrant methylation of tumor suppressor gene *DLC1* promoter was detected in 4/6 HL cell lines, and 44% HL patients. The hyper-methylation of *DLC1* was also viewed as a biomarker of early diagnosis and prognosis. 3p22 cluster genes were demonstrated by numerous studies to have tumor suppressor function in a lot of malignancies; and a member of 3p22 gene, *DLEC1*, was also found to be silenced by methylation at CpG island of the promoter (21). *TET1* belongs to the *TET* family of DNA hydroxylases, and TET proteins are active CpG demethylases and they convert 5-methylcytosine to 5-hydroxymethylcytosine. It was reported to be frequently methylated in HL patient samples, which also supported the tumor suppressor function of TET1 (22). Similar phenomena were also observed in some other tumor suppressor genes such as *RASSF1A* (23), *PCDH10* (24), *PRDX2* (25).

The silence of B-cell specific genes such as *CD79B, BOB1, SYK, PU.1, CD19, CD20* was also found in cHL, and DNA methylation contributes to the silence of these genes (26). Besides, histone acetylation and DNA demethylation of B cells can induce extinction of B cell-specific genes, and result in up-regulation of some HL specific genes. Interestingly, one feature of HRS

cell is the loss of B cell phenotype, suggesting a role of epigenetic alterations in the formation of HL.

Hyper-methylation of several tumor-associated genes such as *P16*, *DAPK*, *GSTP1*, *CDH1* had been found in both EBV positive and negative HL (27), these genes are thought to be involved in cell cycle regulation, apoptosis, and DNA repair, being-important in cancer development. Micro ribonucleic acids (MiRNAs) can function as tumor suppressor genes or oncogenes, miR34a and miR203 are tumor suppressor genes in some cancers, and they were also hyper-methylated in HL cell lines and tissue samples from HL patients (28). Dhiab et al (29) also found that methylation of tumor suppressor microRNA miR9, especially miR9-2, is frequently observed in HL patient samples, irrespective of EBV infection.

2.2 TELOMERE AND TELOMERASE

Telomere is the nucleoprotein structure formed at the end of chromosomes. Telomere and its binding proteins (POT1, RAP1, TRF1, TRF2, RAP1, TPP1) work together to keep the genome stability (30). It is commonly believed to be related to aging in humans, and the shortening of telomere is observed in human tissues as people age (31).

2.2.1 Telomerase

Telomerase is a ribonucleoprotein enzyme that belongs to the reverse transcriptase family. Telomerase reverse transcriptase (TERT) and internal telomerase RNA template (TERC) constitute the core of telomerase to lengthen telomere (32). In most human cancers, telomerase is reactivated by induction of TERT expression. The telomerase activity is detected in hematopoietic cells, germ cells and stem cells. Besides, telomerase activity and the maintenance of telomere are thought to be highly related to the progression of a large number of human malignancies (33). It regulates the progression of cancers mainly through mediating the unlimited division of tumor cells (34). TERC is a non-coding RNA as the template for telomeric DNA synthesis.

Telomerase has long been thought of as a therapeutic target for cancers. Of all the telomerase inhibitors, GRN163L (also named as imetelstat) was thought to be one of the most effective inhibitors. It provides a competitive inhibition effect with telomerase through binding to the template region of telomerase RNA component (Figure 1) (35).

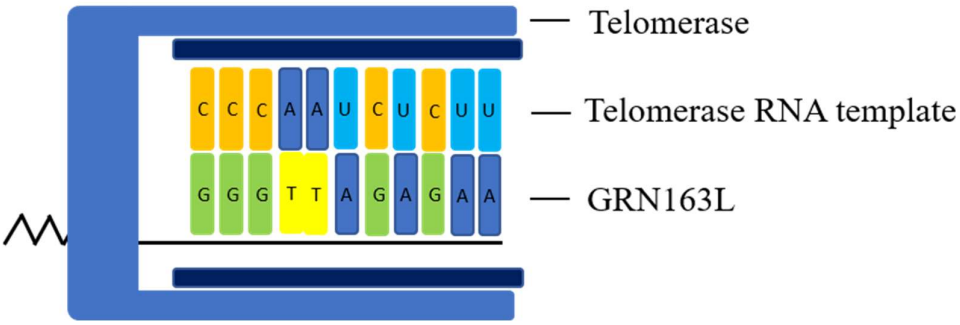

Figure 1. *GRN163L inhibits telomerase activity through the binding of human telomerase RNA template.*

2.2.2 Telomere maintenance mechanism and regulation of telomerase reverse transcriptase (TERT) expression

The telomere maintenance is regulated by TERT reactivation through different mechanisms, including TERT/TERC gene amplification, TERT promoter mutations, epigenetic alterations, transcription factor regulation, alternative splicing, and polymorphic variants (36). The TERT/TERC gene copy number changes in chromosomes result in the alteration of gene expression. TERT promoter mutation is common in the non-coding region, and the most common TERT promoter mutations occur at -124bp and -146bp from the transcription starting site. The mutation is usually C>T transition, and the two hotspot mutations above are so-called C228T and C250T, respectively (37, 38).

The epigenetic regulation is very common in cancers (39), TERT promoter methylation is another kind of regulatory mechanism in the TERT expression, as well as the telomerase activity (40). The promoter of TERT has many binding sites of transcriptional factors that activate or repress the gene expression, and many important signal pathways were reported to be related to the regulation of TERT expression as well as telomerase activity, for example, RAS/RAF/MEK pathway, PI3K/AKT pathway, IKK/NF-κB pathway, JAK-STAT pathway, etc (41). Single nucleotide polymorphisms (SNPs) at the TERT locus have been related to prognosis of many cancers (42-44). The posttranscriptional regulation of TERT was also detected by some research groups, for example, the TERT gene pre-mRNA alternative splicing (36), however, the exact role of this splicing wasn´t completely clarified yet.

2.3 MYELOPROLIFERATIVE NEOPLASMS (MPNS)

MPNs are derived from hematopoietic cells in the bone marrow, and they include three subtypes: polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF). Patients with advanced disease have symptoms including fatigue, weight loss,

weakness, enlarged spleen. The identification of the Janus kinase 2 (JAK2) mutations was found to be related to MPNs in 2005. $JAK2^{V617F}$ mutation is the most important mutation in MPNs and is commonly found in ET, PV, and PMF.

2.3.1 Epidemiology of MPNs

MPNs are believed to be rare hematological diseases, however, the incidence is growing recently. The incidence rate of MPNs roughly varied from 1.15 to 4.99 persons per 100,000 persons per year (45-47). In Sweden, the annual incidence of all MPNs was about 4.45 persons per 100,000 persons from 2000 to 2014 (48).

2.3.2 Janus Kinase 2 (JAK2) inhibitors in the treatment of MPNs

So far, there have been no curable treatment strategies for MPNs, with most treatments focused on the relief of symptoms and control of the proliferation of blood cells. For a small number of patients, hematopoietic stem cell transplantation might be used as a very effective treatment strategy. JAK2 is a kind of non-receptor tyrosine kinase characterized by the lack of Src homology binding domains but with seven Janus homology domains (JH1-JH7). It is essential in many biological processes including cell growth, cancer development, differentiation, immune response, etc. It affects many signaling pathways through binding of type I or type II receptors in cells. JAK2 is involved in the biological actions through activation of MAPK, PI3K as well as AKT pathways (49).

Mutations of JAK2 have been identified in PV, ET, and PMF (50). The most common JAK2 mutation harbours the homozygous G to T transversion and the phenylalanine was substituted for valine at 617 of JAK2 ($JAK2^{V617F}$). The percentage of $JAK2^{V617F}$ mutation varies in patients with ET, PV and PMF. The JAK2 mutation was also thought to contribute to the diagnosis of PV (51). Almost all PV cases were found to carry $JAK2^{V617F}$ mutation, while only 50% of ET and PMF patients were reported to have $JAK2^{V617F}$ mutation (52, 53).

Some JAK2 inhibitors are now in clinical use for the treatment of MPNs. Thus, several drugs were approved by Food and Drug Administration (FDA) or European Medicines Agency for the treatment of MPNs. Ruxolitinib has been approved to treat MF (54). Fedratinib is another JAK2 inhibitor that was approved by FDA for the treatment of intermiediate-2 or higher risk MF patients (55). However, there are some limitations of JAK2 inhibitors in the treatment of MPNs. Because JAK2 inhibitors could not bind specifically to the mutant protein during the treatment, the off-target phenomenon is very common in drug treatment. For example, JAK1 and FLT3 might be affected during the JAK2 inhibition (56). An interesting phenomenon is that JAK2 inhibition is used for both patients with or without JAK2 mutation (57), indicating

a more general effect of JAK2 inhibitors for the treatment of MPNs. However, JAK2 inhibition is not so far a curative therapy.

The combination of JAK2 inhibitors and telomerase inhibitors has been reported to have an effect on the treatment of some MPN patients. According to a clinical trial, for 33 of the intermediate-2 or high-risk MF patients who had GRN163L treatment, 48% of them received prior JAK2 inhibitor treatment. Twenty-one percent of them showed a complete or partial remission (3), supporting a promising future of GRN 163L in the treatment of MPNs.

2.4 LIPOXYGENASE AND ITS METABOLITES

Lipoxygenase (LOX) is a family of enzyme which converts polyunsaturated fatty acid such as arachidonic acid and linoleic acid into mediators playing important roles in asthma, allergic reactions and in the immune system. These mediators act as local hormones in the body. In humans, six *LOX* genes were identified, including *ALOX-5, ALOX-12, ALOX-15, ALOX15B, ALOX12B, ALOXE3*.

2.4.1 5-lipoxygenase (5-LOX) and 5-lipoxygenase-activating protein (FLAP)

The 5-LOX pathway plays an important pathophysiological role in certain diseases, and 5-LOX is the key enzyme in the formation of LTs. Arachidonic acid can be converted to 5-hydroperoxide eicosatetraenoic acid (5-HPETE) and then further converted to LTA_4 under the catalysis of 5-LOX, or transformed to 5-hydroxyeicosatetraenoic acid (5-HETE) by GSH peroxidases (58). In different cell types, LTA_4 is converted to either LTB_4 by LTA_4 hydrolase, or to LTC_4 by LTC_4 synthase, respectively (Figure 2). LTC_4 can be subsequently metabolized into LTD_4, and LTE_4.

5-LOX is found to be highly expressed in many types of leukocytes, such as monocytes, dendritic cells (DC), mast cells and B lymphocytes. The metabolites of the 5-LOX pathway have been implicated in many diseases, such as asthma, allergic disorders, atherosclerosis, Alzheimer´s disease, and some cancers (59-61). In cancer cells, some studies indicate that 5-LOX regulates cell proliferation and cell growth (62). CYSLTR1 antagonists are efficient drugs for the treatment of asthma and some allergic diseases. Zileuton is a potent selectively inhibitor of 5-LOX, and it is used in patients with severe asthma in USA. Some studies indicate that it might also be useful in the treatment of chronic myeloid leukemia (63).

FLAP is a protein that contributes to the cellular production of LTs. It is important in the conversion of endogenous arachidonic acid by 5-LOX and function as the anchor of 5-LOX protein in the membrane. There are some FLAP inhibitors used in clinic trials and scientific

research such as MK886 and GSK2190915. MK886 was first identified by Merck-Frosst in the 1980s, and found to selectively affect LT production (64). GSK2190915 is another potent FALP inhibitor, being tested in the treatment of asthma (65). Some FLAP inhibitors have been in clinical trials for the treatment of asthma and other inflammatory disorders but no one has yet come to the market (66).

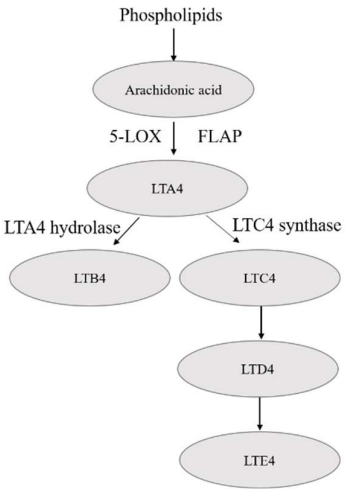

Figure 2. *Schematic diagram of the 5-LOX pathway.*

2.4.2 Leukotrienes

Leukotrienes (LTs) are produced by leukocytes and belong to the eicosanoid family, and are involved in the regulation of the immune system (Figure 3) (67). Exogenous addition of LTB_4, induces the production of IL-2 and, IL-4 in human T cells, and also promotes the proliferation of CD4 T cells. In B-chronic lymphocytic leukemia cells, LTB_4 was found to play a pivotal role in CD40-dependent activation of the cells (68). LTB_4 functions as a chemoattractant and induces the chemotaxis in macrophages, neutrophils and DC (69). The production of LTs causes inflammation mainly mediated by cysteinyl LTs and LTB_4. Cysteinyl-containing LTs include LTC_4, LTD_4 and, LTE_4.

LTB_4 was first reported to be one of the most potent chemotactic agents in 1980 (70), and it was found to promote the migration of leukocytes towards inflammatory tissues (71), increase the phagocytosis (72), and promote the antimicrobial defense (73). Our research group found that arachidonic acid could be converted to LTB_4 in B lymphocytes (74). The mechanism of activation of LT synthesis in B-cells is unclear. It has been known that B lymphocytes can produce LTB_4 in the homogenate and after changing cellular redox status (75), but the formation of LTB_4 has never been reported in B lymphocytes under physiological conditions. For a long time, cysteinyl-containing LTs have been known to play a pathophysiological role

in asthma, and drugs which block Cysteinyl Leukotriene receptor 1 (CYSLTR1) are very commonly used in the treatment of asthma (76). LTB_4 is produced by leukocytes such as neutrophils, B lymphocytes, monocytes, macrophages, eosinophils and mast cells.

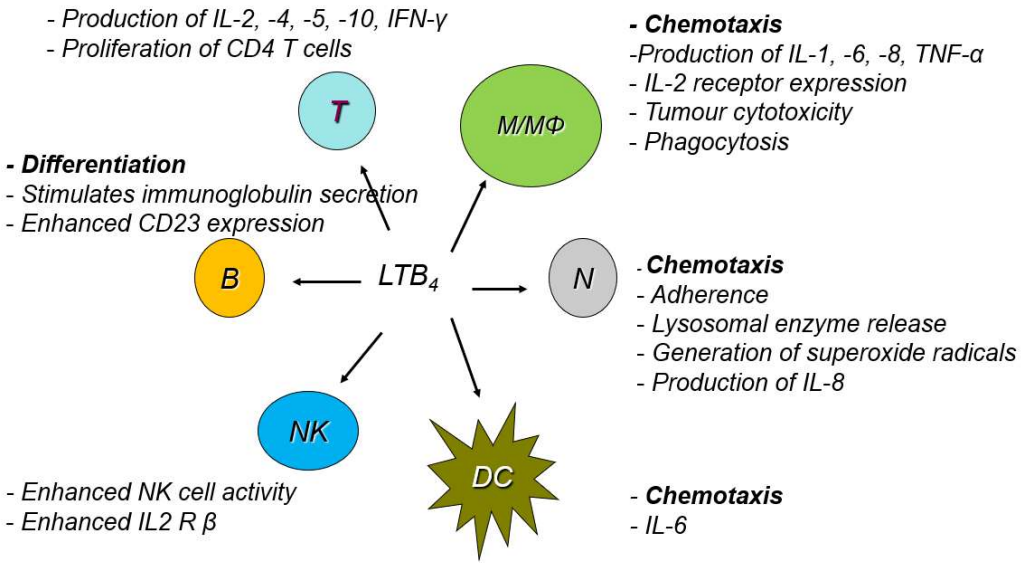

Figure 3. *The effects of LTB_4 in the immune system in the human body.*

2.5 MANTLE CELL LYMPHOMA (MCL)

2.5.1 Epidemiology

MCL belongs to non-Hodgkin lymphoma (NHL), it represents about 6% of all NHL patients, and the incidence of MCL in western countries is about 0.5 person per 100,000 persons per year (77, 78). In western countries, the incidence is found to be higher than in Asian countries (79, 80). However, the incidence of MCL is rising during recent years. Although MCL does occur in young adults, most patients are diagnosed at the age of 60 or 70 (81). About 3/4 of all the MCL patients are male.

2.5.2 Etiology

The MCL risk factors have not been fully clarified yet. However, some studies indicate that the incidence of MCL is believed to be correlated with familial factors, Borrelia infection, polymorphisms in IL-10. In addition, some researchers also found a relationship between autoimmune disease and the incidence and the prognosis of MCL patients. MicroRNAs are also reported to play a role in the etiology of MCL (82).

2.5.3 Pathogenesis

MCL is thought to be caused by the combination of several somatic mutations in cells. The aberrant cell cycles, DNA damage, BCR cell signaling, the lymphoid tissue microenvironment with the cancer tissues contribute to the MCL pathogenesis. The cyclin D1 overexpression, SOX-11 overexpression, *TP53* mutation, *NOTCH1/2* mutations (83) are thought to be related to the pathogenesis of MCL, too.

2.5.4 Treatment of MCL

MCL is a disease with a dismal outcome. The treatment of MCL now still needs great improvement since almost all patients will finally relapse. Nowadays there are four main kinds of treatment strategies, including chemotherapy, immune therapy, radiotherapy, and some novel agents in clinical trials. Chemotherapy is the most commonly used treatment in MCL. However, the treatment with chemotherapy could not be repeated because of some severe side effects such as hematologic suppression. The combination of CHOP and rituximab monoclonal antibody is common in MCL treatment, and patients benefit from the prolonged drug efficacy. Rituximab in combination with thalidomide was also reported to show some effect in the MCL treatment (84). Zanubrutinib and Brexucabtagene autoleucel were recently approved by FDA for the treatment of patients who are refractory to chemotherapy (85, 86).

Ibrutinib is a Bruton protein-tyrosine kinase (BTK) inhibitor binding to BTK and inhibits BTK activity which is essential for B cells, thereby causing the defect of B cell receptor signaling pathway. Ibrutinib could also lead to the interference of CD40 signaling related to the migration of B cells towards the lymph node (87, 88). Ibrutinib is now commonly used in the treatment of patients diagnosed with chronic lymphocytic leukemia, MCL and Waldenstrom´s macroglobulinemia (89). Ibrutinib was approved by FDA in the USA for the treatment of MCL in 2017 (90).

3 STUDY AIMS

Overall, we aimed at exploring the pathogenesis of common hematological malignancies (HL, MPNs, MCL) for the rational development of new therapeutic strategies. Specially in each paper, the study aims are:

1. To explore the role of DNA methylation in the pathogenesis of HL, by analyzing monozygotic triplets.
2. To determine how JAK2 inhibitor LY2784544 affects the $JAK2^{V617F}$ bearing cell line HEL, and explore whether telomerase inhibitor GRN163L could help to gain a better therapeutic effect in MPNs.
3. To explore the role of 5-LOX pathway in the migration and adherence of malignant B cells.

4 MATERIALS AND METHODS

4.1 PATIENT SAMPLES (PAPER I)

The triplet blood samples were collected at the same time at the Department of Hematology, Karolinska Hospital. These studies were approved by the ethics committee of Karolinska Institutet. All the experiments were performed according to the guidelines of Karolinska Institutet. Informed consent was obtained from all participants before these studies.

4.2 CELL LINES AND CELL CULTURE (PAPERS II, III)

Human erythroleukemia cell line HEL was used in paper II, and MCL cell line JeKo-1, T cell line Jurkat, Molt-3 cell lines, and human stromal cell line HS-5 were used in paper III. All cells were cultured at 37°C. RPMI or DMEM supplemented with 10% FBS, 1U/ml PenStrep and 1% L-glutamine was used as the culture medium.

4.3 WESTERN BLOT (WB) ANALYSIS (PAPERS II, III)

Cell pellets were extracted with RIPA lysis buffer (Thermo Scientific, USA). Protein concentration was detected by Bicinchoninic acid assay (BCA) kit (BIO-RAD, USA), and same amount of protein was loaded into wells of mini-protein TGX gels (BIO-RAD, USA) followed by protein transfer to polyvinylidene difluoride (PVDF) membranes (BIO-RAD, USA). The membrane was blocked with 5% milk for one hour at room temperature, and the membrane was incubated with 5-LOX or KLF4 antibodies overnight at 4°C. Goat anti-rabbit or mouse secondary antibodies were used after primary antibody incubation. Membrane was developed after activation with ECL substrate (BIO-RAD, USA).

4.4 RNA EXTRACTION AND Q-PCR ANALYSIS (PAPER II)

Total RNA was extracted using Trizol (Life technologies) according to the manufacture´s instruction. RNA quantitation was detected by Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers. Quantitative real-time polymerase chain reaction (q-PCR) was carried out with SYBR (Appliedbiosystems, UK) and specific primers. Primers used for q-PCR are listed below in table 1.

Table 1. *Primer sequences.*

	Forward	Reverse
β-2M	5'-GAATTGCTATGTGTCTGGGT-3'	5'-CATCTTCAAACCTCCATGATG-3'
KLF4	5'-TCAGCGTCAGCCTCCTCTT-3'	5'-TTGATGTCCGCCAGGTTGAA-3'
TERT	5'-CGGAAGAGTGTCTGGAGCAA-3'	5'-GGATGAAGCGGAGTCTGGA-3'

4.5 CELL TREATMENT (PAPERS II, III)

In paper II, HEL cells were treated with JAK2 inhibitor LY2784544 (working concentration is 0.5 μM) and the specific telomerase inhibitor GRN 163L (working concentration is 20 μM). In Paper III, FLAP inhibitor GSK2190915, MK886, 5-LOX inhibitor zileuton, and BTK inhibitor Ibrutinib were used in the treatment of cell line JeKo-1, Jurkat, Molt-3. GSK2190915, MK886, Ibrutinib, and zileuton were used starting from the concentration of 0.1 μM to 1 μM. The structure of all compounds is listed below in Figure 4.

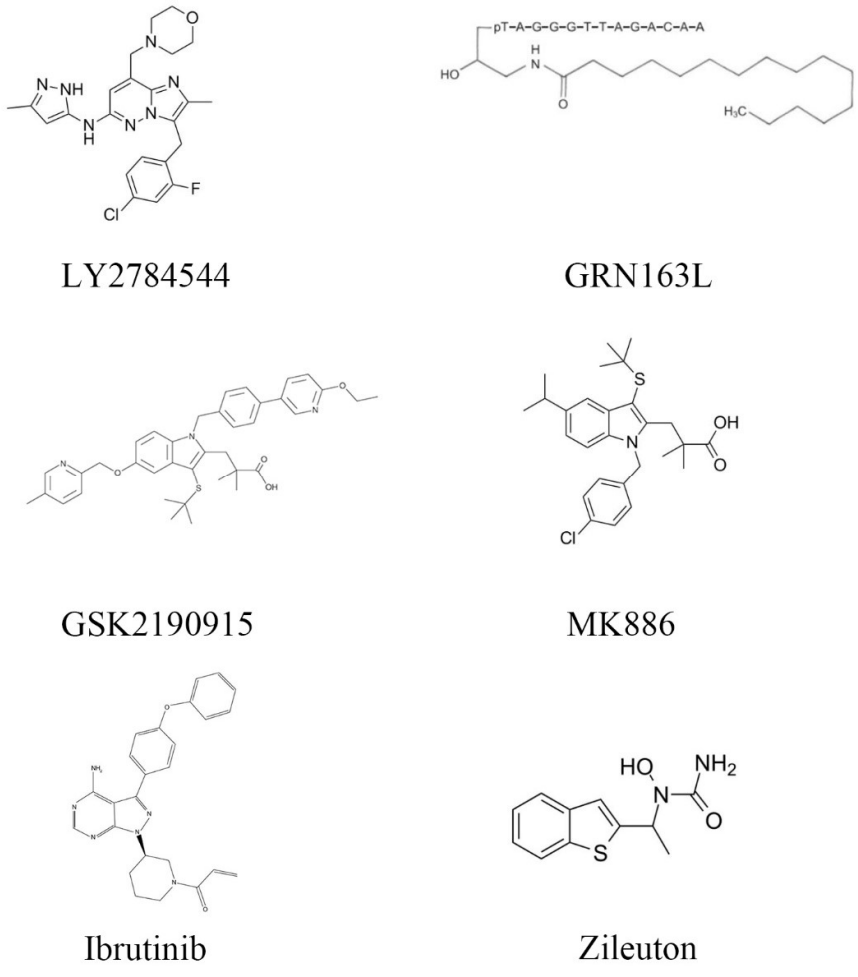

Figure 4. *Chemical structures of compounds.*

4.6 CD34+ CELL AND B-CELL SUBPOPULATION ISOLATION (PAPERS I, II)

CD34+ subpopulations and CD34+ HEL cells were isolated with CD34+ isolation kit. B-cells were sorted from the PBMCs using CD20+ positive selection isolation kit. CD20+ cells were labeled with CD19 (APC-Cy7), IgD (FITC) and CD27 (PE) antibodies (BD). Naïve (IgD+ CD27-), marginal zone-like (IgD+ CD27+) and switched memory (IgD- CD27+) B-cells were sorted using FACS Aria (BD).

4.7 DNA METHYLATION ANALYSIS (PAPER I)

Genomic DNA was isolated from CD34+ cells, Marginal zone-like B-cells, Naïve B-cells, and switched memory B-cells with the DNeasy Blood & Tissue Kit (Qiagen). Bisulfite conversion was performed using the EZ DNA MethylationTM Kit D5004 (Zymo Research), and the bisulfite converted DNA was eluted in 15µl according to the manufacturer´s protocol. DNA methylation of genomic DNA was performed with Illumina EPIC array.

4.8 MIGRATION ANALYSIS (PAPER III)

JeKo-1 cell line, Jurkat cell line and Molt-3 cell line were seeded in 12-well plates. After inhibitor incubation in serum-free medium (SFM) for four hours, cells were collected and transferred to the upper chamber of 6.5 mm transwell plates with 8 µm pore (Corning Incorporated, USA) for two hours. CXCL12 was used in the lower chamber as the chemo-attractant, and the cells in the lower chamber were collected and counted with Burker chamber.

4.9 COLONY FORMATION ANALYSIS (PAPER II)

HEL cells pretreated with JAK2 inhibitor LY2784544 for 4/8 weeks and telomerase inhibitor GRN163L for 4/8 weeks were used for the colony formation analysis. Five-hundred cells/well were seeded in a 6 well plate cultured with Methocult H4100 and RPMI 1640. Methocult H4100 was used for the formation of growth of leukemic progenitor cells. After 10 days of cell culture, cell colonies that contain 25-50 cells or more than 50 cells were counted.

4.10 FLOW CYTOMETRY (PAPERS I, II, III)

In paper I, the isolation of sub-B populations was performed with flow cytometry. In paper II, Annexin V-FITC/7-AAD kit (Beckman coulter) was used for the detection of cell apoptosis. In paper III, JeKo-1 cells adherent to HS-5 cells were subjected to Flow cytometry analysis.

4.11 TELOMERASE ACTIVITY ASSAY (PAPER II)

The telomerase activity was detected by telomeric repeat amplification protocol (TRAP) ELISA KIT (Roche, Switzerland). Telomerase elongated the 3′ end of a biotin-labeled synthetic primer with 1µg lysate of HEL, the product was amplified with initial PCR under the condition of 25 cycles. The amplified products were denatured and hybridized to digoxigenin (DIG)-conjugated detection probes specific to the telomeric sequence. The products were then affixed to a streptavidin-coated microplate by the biotin. Amplified products stuck to the microplate were bound to antibodies against DIG, conjugated with horseradish peroxidase and the peroxidase substrate. The level of telomerase activity was expressed as absorbance determined with an ELISA reader.

4.12 TELOMERE LENGTH ANALYSIS (PAPER II)

Flow-FISH was used for the measurement of telomere length. The fluorescent PNA probes were hybridized to the telomere sequence and the signal was detected by Gallios flow cytometer. Kaluza software was used for the analysis. Fluorescent MESF-FITC beads were used and the QuickCal v2.3 data analysis program was used for the quantification of the fluorescent signal. The Telomere length was calculated according to this formula:

Telomere length, kB = MESF x n_{base}/ n_{chr} x 1000, where MESF is the fluorescent intensity given by QuickCal v.2.3, n_{base} is the number of bases per PNA probe and n_{chr} is the number of chromosomes of HEL cells.

4.13 LENTIVIRAL TRANSFECTION (PAPER II)

KLF4 shRNA lentiviral particle was used for the lentiviral transfection. HEL cells were seeded at a density of 0.4 million/ml cultured with RPMI 1640 supplemented with 10% FBS, 1mg/ml L-glutamine and 1mg/ml penicillin/streptomycin. HEL cells were infected with the virus together with 5 µg/ml Polybrene. After 16 hours′ infection, the medium was changed followed by puromycin selection at a concentration of 1 µg/ml.

4.14 CELL-BINDING ASSAY OF JEKO-1 CELLS (PAPER III)

Human stromal cell line HS-5 and JeKo-1 cell line was cultured with RPMI supplemented with 10% FBS, 100U/ml penicillin and 100 mg/mL streptomycin. JeKo-1 cells were labeled with Cell Tracer Carboxyfluorescein succinimidyl ester (CFSE, C34554 ThermoFisher), and HS-5 cells were labeled with Far-red (C34564 ThermoFisher) according to instructions of the manufacturer. SFSE labeled JeKo-1 was added to the HS-5 monolayer in the plate at 10:1 ratio.

After four hours of co-culture, adherent JeKo-1 cells were kept on the monolayer of HS-5 after trypsinization for 1 minute and PBS washing. For treatments, JeKo-1 cells after staining with CFSE were treated with compounds for one hour and then co-cultured with monolayer HS-5 cells for four hours in the absence or presence of different concentrations of compounds and then cells were subjected to Flow cytometry analysis.

4.15 ALOX5 KNOCKOUT WITH CRISPR/CAS9 (PAPER III)

ALOX5 gene deletion in JeKo-1 cells was performed using CRISPR/Cas9 technique. Two different guide RNAs targeting ALOX-5 locus (AGATGGATGGAGTGGAACCC-TGG and CTATCAAACTGGATATCACG-GGG) (CRISPR evolution sgRNA EZ Kit, Synthego) and Cas9 protein (Alt-R® S.p. Cas9 Nuclease V3, IDT) were pre-complexed in ribonucleoproteins (RNPs). The RNPs were then introduced into the cells through electroporation with the Neon system (ThermoFisher Scientific) (1500V, 20 ms, 1pulse). The guides were designed against exon 4 of the ALOX-5 locus. The *ALOX5* gene downstream ATG at exon 4 was mutated to avoid potential initiation.

4.16 WHOLE TRANSCRIPT EXPRESSION ANALYSIS (PAPER II)

To analyze the mechanism of CD34 cell accumulation after JAK2 inhibition, HEL cells treated with LY2784544 and/or GRN163L were analyzed with whole transcript expression analysis. Microarray assay was performed with Affymetrix whole-transcript expression analysis and the WT assay gene ST1.1 (Affymetrix, Santa Clara, CA, USA) according to the manufacture´s protocol and the Bioinformatic and Expression Analysis Core Facility (BEA), Karolinska Institutet.

4.17 STATISTICAL ANALYSES

IBM SPSS Statistics 24 software was used for the statistical analysis. One-way ANOVA test was used to compare the migration difference of different cell lines, and student t-test was used for JeKo-1 cell adherence assay, the results of TRAP, mRNA expression, colony formation assay, and flow-FISH of TL. P-values <0.05 were considered statistically significant.

5 RESULTS

5.1 PAPER I DNA METHYLATION DIFFERENCES IN HL TRIPLETS

Four cell subtypes were examined in our study: CD34+ cells, Naïve B-cells, marginal zone-like B-cells, and switched memory B-cells. Our results showed that four cell subpopulations were different from each other regarding the methylation status, as shown in Figure 5A. When we compared the HL triplets´ methylation status with the HL free triplet in the CD34+ cells and B cell subpopulations, shared methylation differences in naïve B-cells and marginal zone-like B-cells were identified (Figure 5B). However, in CD34+ cells and switched memory B-cells, no common methylation difference was detected. In the naïve B-cells and marginal zone-like B-cells, all the methylation displayed the hyper-methylated status when compared with the HL free triplet.

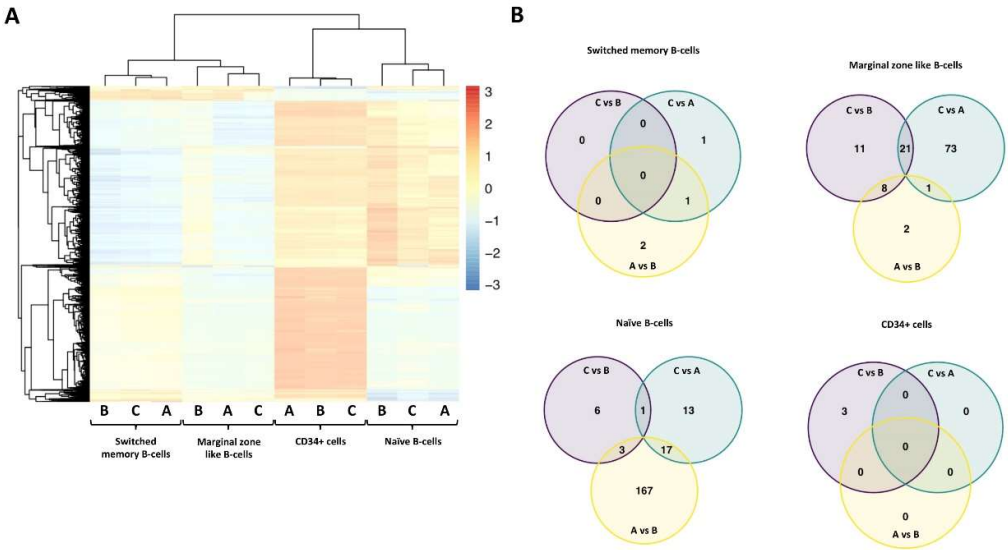

Figure 5. *A. Unsupervised hierarchical clustering of methylation levels from the 10 000 most variable probes across all samples derived from the triplets. B. Overlaps of different methylated regions when each triplet is compared to the others in pairwise comparisons.*

Of all the regions screened in these four cell subtypes, we found that one region on chromosome 18 differed in methylation in the naïve B-cells, where the gene *CDH19* was located. In marginal zone-like B-cells, we detected 21 shared genomic regions on 10 different chromosomes. The corresponding genes and the probe regions were listed in Table 2.

Table 2. *Genes with shared methylation differences in HL affected triplets compared with HL free triplet.*

Cell type	Chr	Start	End	Length (bp)	Gene / Symbol of overlapping transcripts (within 10 000 bp)
Naïve B - cells	18	64271341	64271643	303	*CDH19*
Marginal zone-like B-cells	1	200860363	200860537	175	*C1orf106*
	1	225117389	225117464	76	*DNAH14*
	2	46465	46752	288	*FAM110C*
	3	3841043	3841252	210	*LRRN1, SUMF1*
	3	129693370	129693791	422	*TRH, RP11-93K22.6*
	3	138067053	138067623	571	*MRAS*
	4	11430353	11430903	551	*HS3ST1*
	4	55991818	55991943	126	*KDR*
	4	77227489	77227631	143	*FAM47E-STBD1, CCDC158*
	4	90758207	90758537	331	*RP11-67M1.1, SNCA*
	5	9546271	9546404	134	*SNHG18, SNORD123, SEMA5A*
	5	128300967	128301185	219	*SLC27A6*
	8	26371428	26371573	146	*BNIP3L, DPYSL2, PNMA2*
	15	35047023	35047411	389	*RP11-814P5.1, GJD2*
	15	57668539	57668554	16	*CGNL1*
	20	13200931	13200992	62	*ISM1*
	22	20792143	20792535	393	*SCARF2, KLHL22*
	X	101905837	101906537	701	*RP4-769N13.6, GPRASP1, RP4-769N13.7*

5.2 PAPER II

5.2.1 The viability of HEL cells decreases after JAK2 inhibition and telomerase inhibition.

The treatment of the specific JAK2 inhibitor LY2784544 at a concentration of 0.5 μM led to the decreased cell number, as well as cell viability when compared with the control group in erythroleukemia cell line HEL. However, the viability of HEL cells started to increase after several weeks of LY2784544 treatment and finally restored to the same level as control group in week 9. In cells treated with telomerase inhibitor GRN163L, no viability recovery was observed. When HEL cells were inhibited with both 0.5 μM LY2784544 and 20 μM GRN163L, no viability and apoptosis recovery was seen with the time after several weeks of treatment (Figure 6).

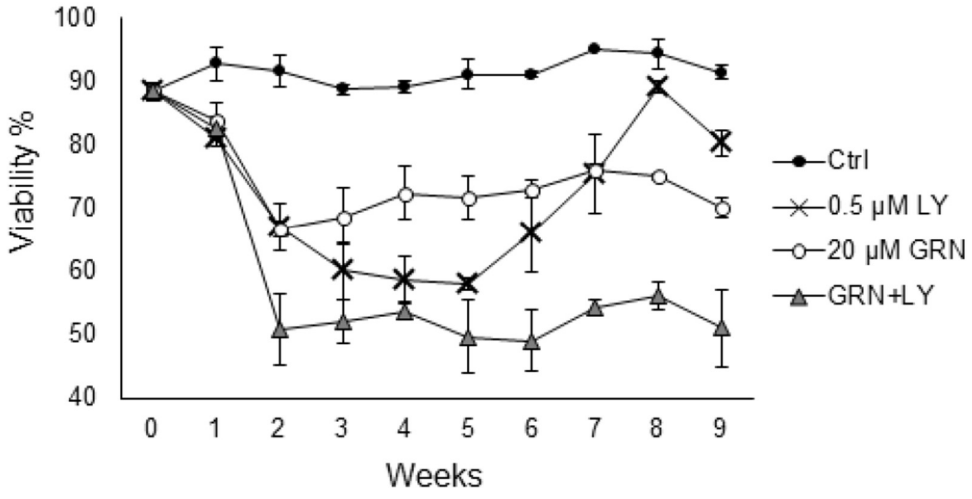

Figure 6. *The viability of HEL cells after treated with LY2784544 and GRN163L, from week 1 to week 9.*

5.2.2 CD34+ HEL cells accumulate after JAK2 inhibition.

In LY2784544-treated group, the fraction of CD34+ HEL cells increased from 10% to about 85% in week 9 (Figure 7A and 7C). However, in the control group, the CD34+ cells decreased over time. Combination treatment of LY2784544 and GRN163L did not induce the accumulation of the CD34+ cells compared with the control group and GRN163L treated group as shown in Figure 7B. After separation of the CD34+ and CD34- HEL cells, the viability and apoptotic cells were less in CD34- cells when compared with the CD34+cells after JAK2 inhibition. More interestingly, in CD34- HEL cells, the percentage of CD34+ HEL cells increased after LY2784544 incubation with time. Colony assay results also showed that cells treated with LY2784544 had potent capacity of forming more colonies (Figure 7D).

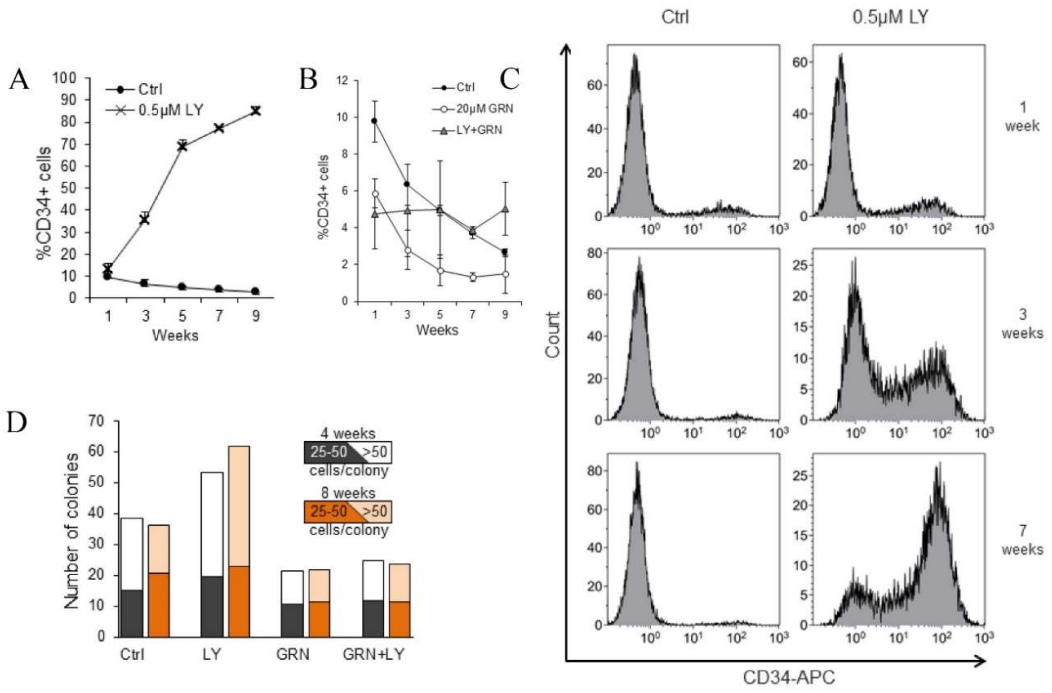

Figure 7. *A. Fraction of CD34+ cells after LY2784544 treatment. B. Fraction of CD34+ cells after GRN163L and the combination treatment. C. Histograms of CD34-APC in HEL cells after LY2784544 treatment. D. Number of colonies after LY2784544 and GRN163L treatment.*

5.2.3 Silencing KLF4 expression reduces the LY2784544-mediated accumulation of CD34+ cells

We performed the whole-transcript analysis of HEL cells pre-treated with JAK2 inhibitors, the gene KLF4 was selected for further analysis. It belongs to SP1-like family and was reported to regulate the stem cells in many features such as proliferation, apoptosis, etc. We found KLF4 was upregulated, as confirmed by WB (Figure 8A). The inhibition of KLF4 was also identified after the combination treatment of JAK2 inhibitor LY2784544 and telomerase inhibitor GRN163L compared with the LY2784544 treatment alone. The lentiviral transfection was set up to knock down the expression of KLF4 through shKLF4 (Figure 8B and 8C). The accumulation of CD34+ cells mediated by LY2784544 treatment was attenuated after KLF4 knock-down (Figure 8D).

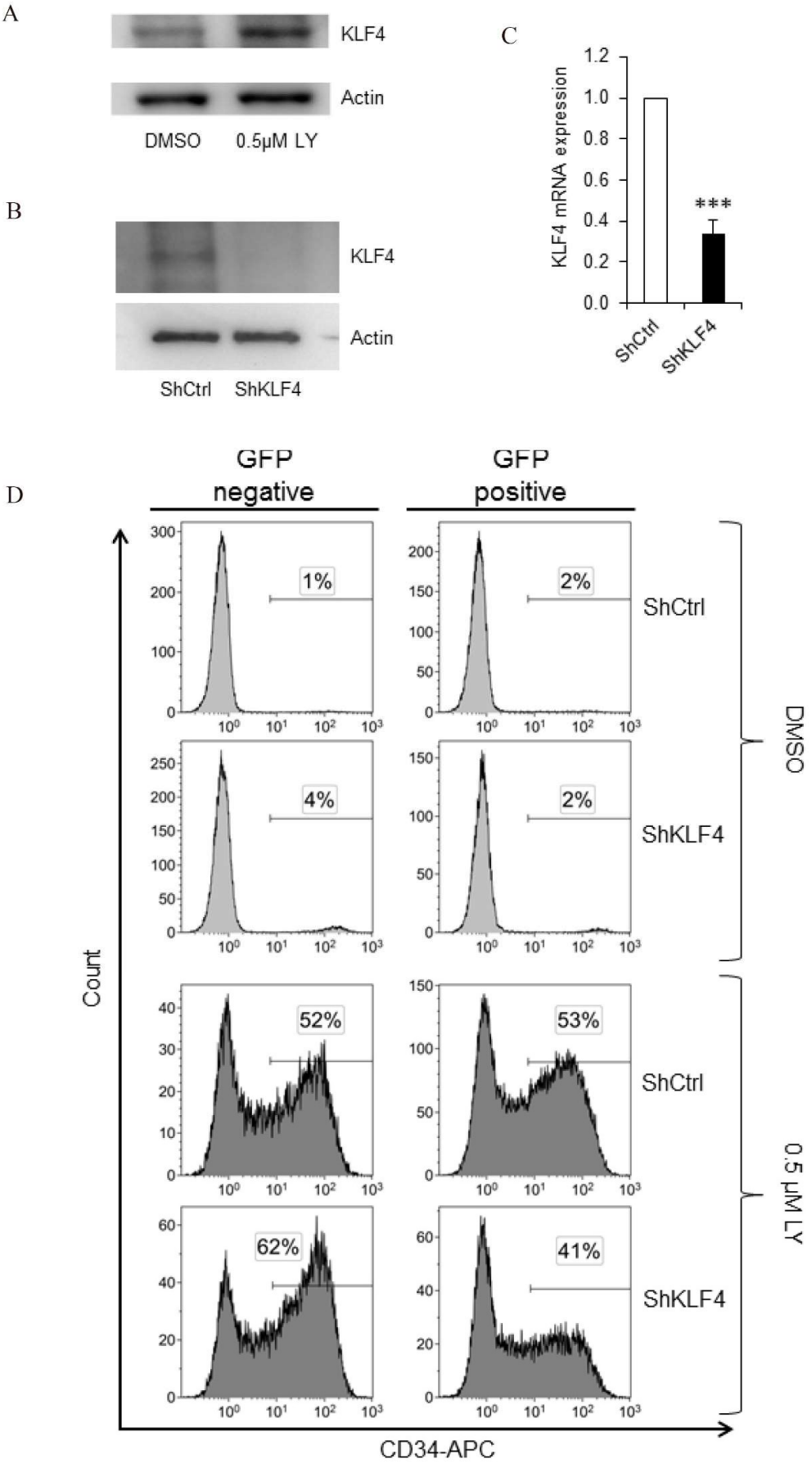
A
KLF4
Actin
DMSO
0.5μM LY
B
KLF4
Actin
ShCtrl
ShKLF4
C
KLF4 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
ShCtrl
ShKLF4
D
GFP negative
GFP positive
1%
2%
ShCtrl
4%
2%
ShKLF4
DMSO
52%
53%
ShCtrl
62%
41%
ShKLF4
0.5 μM LY
Count
CD34-APC

Figure 8. *A. KLF4 protein expression after LY42784544 treatment. B. KLF4 protein expression after knocking down with ShKLF4. C. mRNA expression after KLF4 knock down. D. Histogram of CD34-APC in HEL cells after two weeks of LY2784544 incubation.*

5.2.4 JAK2 inhibition leads to the down-regulation of TERT and telomerase activity, but elongates telomere length.

The decreased telomerase activity and TERT expression was detected after JAK2 inhibition compared with the control group. However, the telomere length was found to increase after LY2784544 incubation in week 7 and 9 (Figure 9A-B). This phenomenon was seen both in CD34+ and CD34- HEL cells, but telomere lengthening was more obvious in CD34+ cells. The longer the incubation time was, the more significant longer telomere length was, which was more significant in week 8 than week 4. The decreased telomerase activity was seen also in both CD34+ and CD34- HEL cells after LY2784544 treatment as shown in Figure 9C-D.

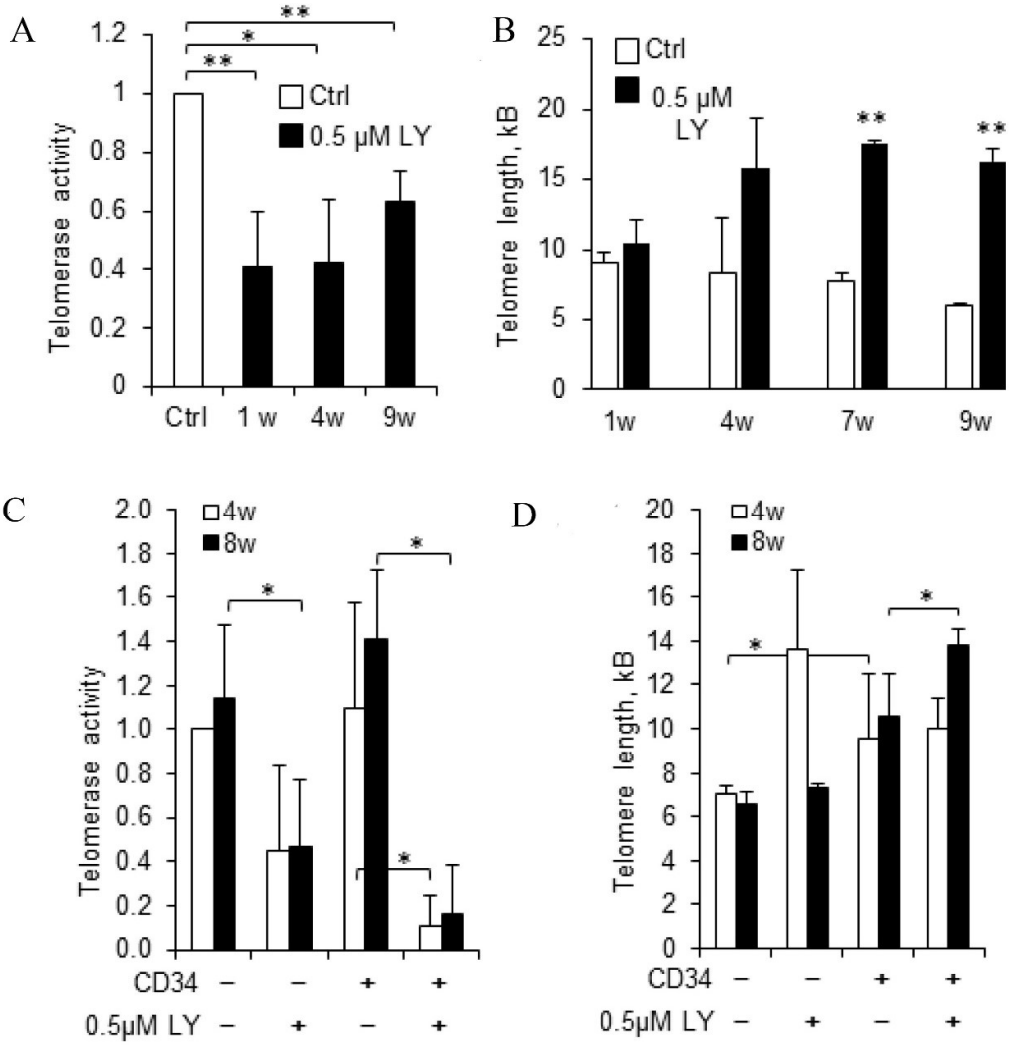

Figure 9. *A. Telomerase activity of HEL cells after LY2784544 treatment. B. Telomere length of HEL cells after LY2784544 treatment. C. Telomerase activity of CD34- and CD34+ HEL cells after LY2784544 treatment. D. Telomere length of CD34- and CD34+ HEL cells after LY2784544 treatment.*

5.3 PAPER III

5.3.1 5-LOX/FLAP inhibitors inhibit the migration of JeKo-1 cells

In human bodies, the spread of MCL cells in the vessels could result in many severe disease-related symptoms. MCL cells contain a large amount of 5-LOX and mediate the production of the chemotactic LTB_4. It was therefore of interest to determine whether the inhibition of 5-LOX has an effect on the migration of these cells. We performed experiments to analyze whether 5-LOX and FLAP inhibitors influence the migration of MCL cells upon stimulation with the potent chemoattractant CXCL12. In our study, transwell assays were used for determination of the migration ability. As shown in Figure 11B, the MCL-derived JeKo-1 cells contain high large amounts of 5-LOX protein (91). The number of migrated JeKo-1 cells increased towards CXCL12 compared with JeKo-1 cells exposed to only SFM (Figure 10A). The migration of JeKo-1 cells towards medium which contains 20% FBS is also shown in Figure 10A. Inhibition of LT synthesis by the FLAP inhibitor GSK 2190915 (GSKi) or the 5-LOX inhibitor zileuton results in the dose-dependent decreased migration of the cells upon stimulation with CXCL12. The migration of cells towards CXCL12 decreased significantly at the concentration of 0.5 μM of the inhibitors. To further determine whether the effect of the inhibitors was mediated by 5-LOX protein, we examined the effect of the inhibitors in 5-LOX negative cells. The T-cell line Jurkat expresses 5-LOX but not FLAP (92). As shown in Figure 10B, LT biosynthesis inhibitors did not inhibit migration of these cells. Similar results were obtained with the T-cell line Molt-3 which does not express 5-LOX (92) (data not shown). Since LTB_4 is a strong chemoattractant for neutrophils and T cells (93), we investigated whether LTB_4 was a chemoattractant for JeKo-1 cells. However, LTB_4 did not stimulate the migration of these cells (the working concentration varies from 0.1μM to 10μM, and data not shown).

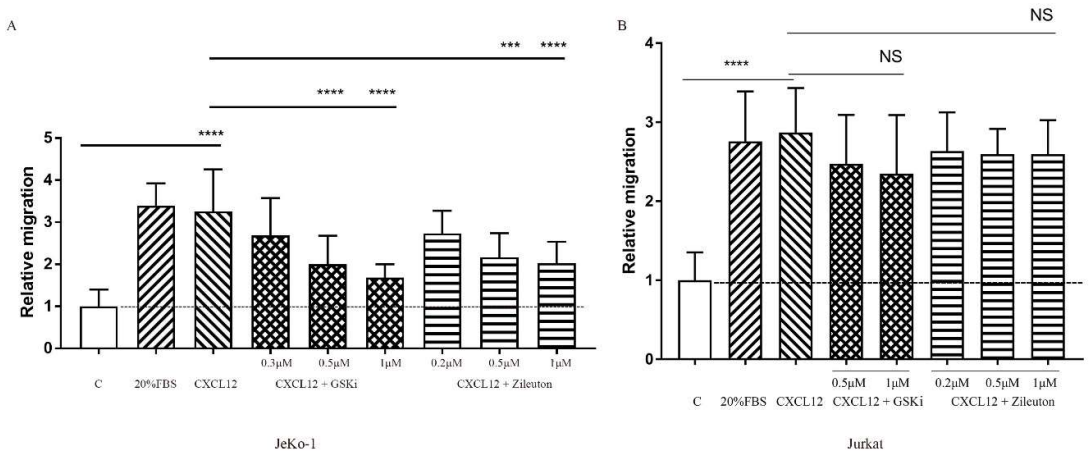

Figure 10. *Effect of GSK 2190915 (GSKi) and Zileuton on the migration of JeKo-1 cells (A) Jurkat cells (B).*

5.3.2 Knocking out ALOX5 gene through CRISPR/Cas9 decreases the migration of JeKo-1 cells.

By using the gene knockout technique CRISPR/Cas9, we established a stable *ALOX5* gene negative JeKo-1 cell line (5-LOX KO) to further probe whether 5-LOX has an important role in migration of these cells. The absence of 5-LOX protein expression of ALOX5 in JeKo-1 cells is shown in Figure 11B. The spontaneous migration in serum-free medium (SFM) or upon stimulation with CXCL12, were significantly decreased in 5-LOX negative JeKo-1 cells in comparison with wild type JeKo-1 cells (Figure 11A).

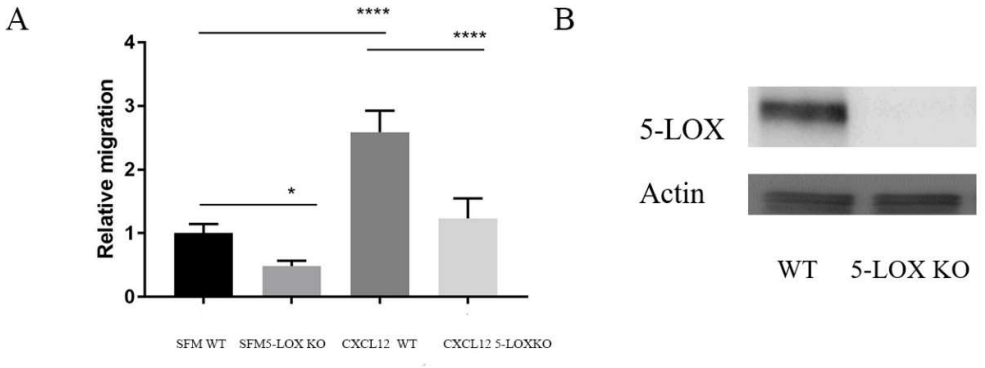

Figure 11. *A. Migration of wild type JeKo-1 cells and ALOX5 KO cells after stimulation with CXCL12. B. Protein expression of 5-LOX after ALOX5 deletion.*

5.3.3 5-LOX/FLAP inhibitors decrease the adhesion of JeKo-1 cells to stromal cells

The adhesion ability of malignant B-cells to stromal cells in bone marrow, lymph nodes and other tissues is also an important pathophysiological event in the spreading of malignant B-cells to other tissues or organs in the body. JeKo-1 cells could adhere to stromal cells (HS-5) (94). Since LTB_4 mediates the stimulation of neutrophils adherent to the vessel wall (93), we explored the effect of 5-LOX and FLAP inhibitors on the adherence of JeKo-1 cells to the human stromal cell line HS-5 (Figure 12). JeKo-1 cells were firstly incubated with these inhibitors for 1 hour and then cells were cultured together with stromal cells for another 4 hours. FLAP inhibitors GSK2190915 (GSKi) and MK886, and the 5-LOX inhibitor zileuton could significantly decrease the number of adherent JeKo-1 cells, starting from the concentration of 0.2μM. These compounds were as potent as ibrutinib regarding the inhibition of adherence of the cells. Our study showed a dose-dependent inhibition effect when cells were treated with MK886 and zileuton, while the maximum effect was shown already observed at the concentration of 0.2 μM for GSKi and ibrutinib. Cell viability was higher than 97% in all experiments (data not shown).

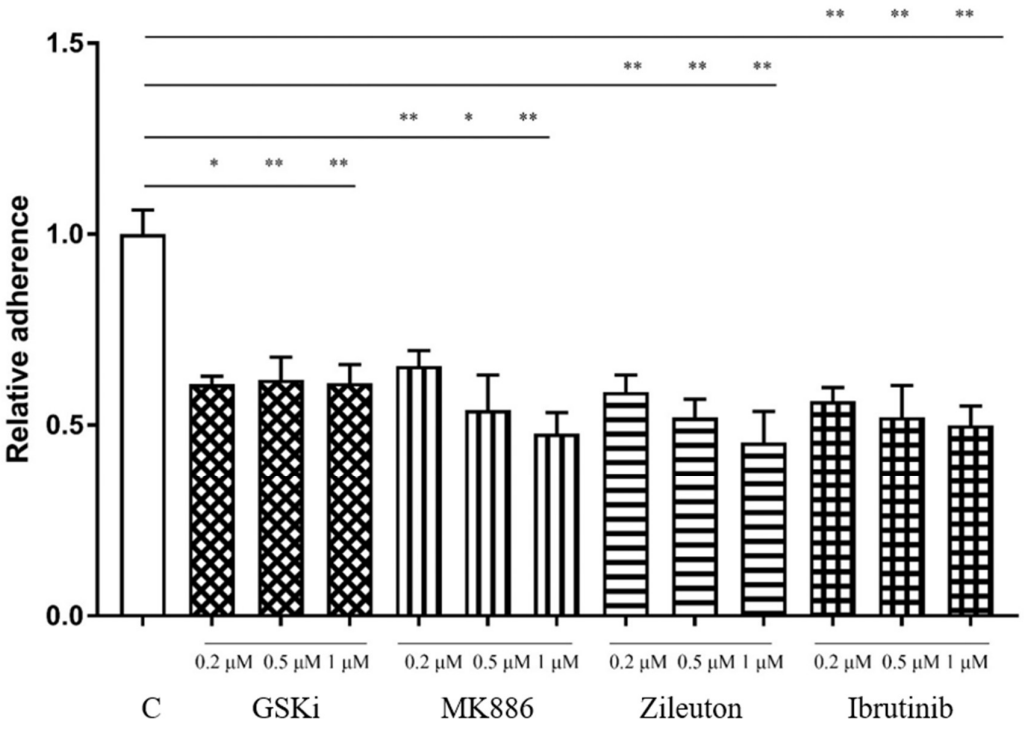

Figure 12. *Relative adherence of JeKo-1 cells to HS-5 after different concentrations of compounds treatment.*

5.3.4 Effect of 5-LOX/FLAP inhibitors on the cell surface expression of CXCR4 in JeKo-1 cells

The chemotactic agent CXCL12 mediates mainly its effect through binding to the CXCR4 receptor on the target cell. We investigated whether 5-LOX/FLAP inhibitors influenced the expression of CXCR4 in JeKo-1 cells. However, neither 5-LOX or FLAP inhibitors nor ibrutinib showed any effect on the expression of CXCR4 in JeKo-1 cells (Figure 13)

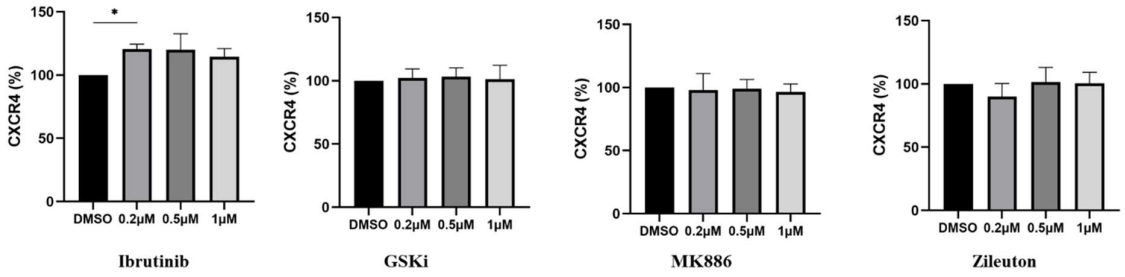

Figure 13. *Relative surface expression of CXCR4 in JeKo-1 cells after treatment with different compounds.*

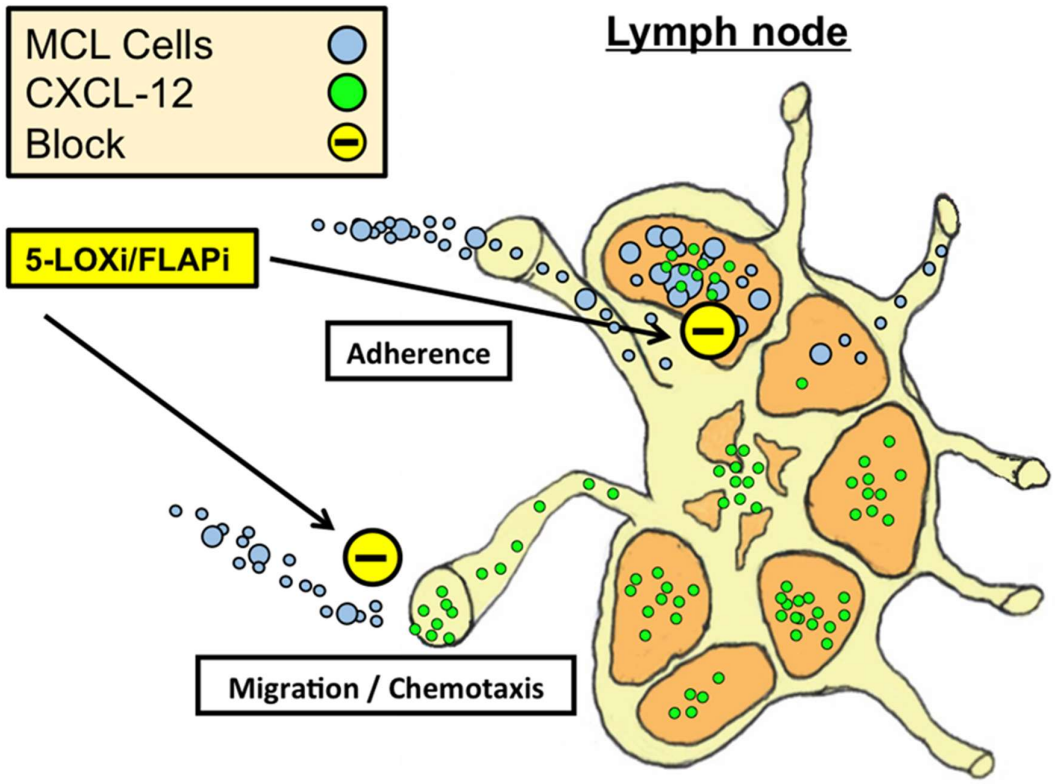

Figure 14. *Theoretical model of the effects of 5-LOX/FLAP inhibitors on the migration and adherence of MCL cells in a lymph node.*

6 DISCUSSION AND CONCLUSIONS

6.1 DNA METHYLATION DIFFERENCES IN HL MONOZYGOTIC TRIPLETS

In this paper (I), we determined the methylation differences of PBMCs from three monozygotic triplets, of which two were diagnosed with HL, the other one is HL-free. The methylation analysis was performed in four subtypes of cells, CD34+ stem cells and three B cell-subpopulations (naïve-B cells, marginal zone-like B-cells, switched memory B-cells). We identified the shared DNA methylation differences in naïve B-cells and marginal zone-like B-cells. Some of the genes in the shared methylated regions were related to the development of cancer or hematopoiesis in humans and animals. For example, ISM1 was reported to be expressed in the lymphocytes, bone marrow, or embryonic blood islands. In zebrafish, it was related to hematopoiesis, especially in the production of hematopoietic stem and progenitor cells (95). KLH22 could promote the tumor-genesis through the regulation of mTORC1, and the depletion of *KLH22* in breast cancer cells inhibits the tumor development in mice models (96). Small nucleolar RNA host gene 18 (*SNHG18*), recently reported to play as a tumor suppressor in hepatocellular cancer, could also be potentially used as the diagnostic biomarker for hepatocellular carcinoma (97). The lncRNA SNHG18 also regulates the cell motility in Glioma (98). MRAS could function as a key signal transducer, and regulate the tumor growth through activation of many important signal pathways such as MAPK and ERK pathways (99). KDR was known to be related to angiogenesis, vascular development and embryonic hematopoiesis. The mutation (p.A 1065T) of *KDR* gene has the potential to promote the tumor proliferation (100).

The mechanism causing the methylation difference between HL affected triplets and the HL free triplet is unknown. The difference might be due to the inter-individual variation, environmental factors, living habits between them or some stochastic events. Since both of the HL-triplets got chemotherapy treatment when they were diagnosed at the age of 40 and 63, respectively, we can not exclude the potential effect of chemotherapy on the methylation differences in these triplets. Some studies demonstrated that chemotherapy could affect the epigenetic pattern. In one study, the 4-month chemotherapy treatment was found to change the DNA-methylation in breast cancer patients (101). In that study, CpG islands in or close to the genes *SDK1, SUF2, VMP1 and CORO1B* were found to be changed after the chemotherapy. After comparison with the genes we screened, none of the above genes were found to be hypermethylated in our study. Interestingly, we found that the *SUMF2* paralog *SUMF1* was altered in marginal zone-like B-cells from both HL triplets. It has been reported that SUMF2

inhibits the suphatase-enhancing activity of SUMF1 (102). We will investigate if this is the case in the HL triplets.

The DNA hyper-methylation we observed in this case probably could lead to the low expression of these genes. Some studies suggest that the inherited-associated somatic mutations might show a low effect on clonal hematopoiesis, which indicates the higher impact of DNA methylation in clonal hematopoiesis (103, 104). It would be of great interest to investigate the epigenetic pattern, especially the DNA methylation in the future in HL and other hematological diseases.

In summary, we here report the DNA methylation differences in three unique monozygotic HL triplets. Some CpG islands of the DNA isolated from marginal zone-like B-cells and naïve-B cells were found to be hypermethylated in HL triplets compared with the HL free triplet, which may have a general implication in the pathogenesis of HL.

6.2 MUTATION OF JAK2^{V617F}-BEARING LEUKEMIA CELLS THROUGH JAK2 INHIBITION ACCUMULATES CD34+ LEUKEMIC STEM CELLS THAT ARE ABOLISHED BY THE TELOMERASE INHIBITOR GRN163L

JAK2^{V617F} mutation is important in driving MPN development. JAK2 inhibitors are now established for MPN treatment. JAK2 inhibition exhibited certain positive effects but is unable to eradicate the disease clone (105). Our findings suggest that the apoptosis and proliferation of HEL cells that carry JAK2^{V617F} could be affected by the JAK2 inhibitor LY2784544 during the first week of treatment, but these effects could be diminished with longer inhibitor treatment. CD34+ cell accumulation may be the reason causing this phenomenon. We found that the stem cell factor KLF4 could be upregulated by the LY2784544 treatment.

KLF4 was reported to induce the production of iPSCs by combining with other three transcriptional factors (Oct3/4, Sox2, c-Myc) (106). However, how KLF4 is acting in hematopoiesis, is not fully clarified. It may be related to the de-differentiation indicating the role of KLF4 in the maintenance of tissue-specific stem cells. We found that the silence of KLF4 attenuated the LY2784544 mediated CD34+ cells´ enrichment, but it was not totally blocked. Similar to our study, KLF4 was found to be down-regulated in CD34+ cells from PV patients, and KLF4 was upregulated followed by JAK2^{V617F} inhibition (107). Our findings and other reports suggested KLF4 was a potential target in the treatment of MPNs. JAK2/STAT pathway was already reported to regulate the TERT expression through binding to

STAT3/STAT5 at the promoter region in human hematological malignancies (41). This suggests a close relation between JAK2 and TERT in the development of malignancies. In our studies, we found that LY2784544 decreased the expression of TERT as well as telomerase activity. But the telomere length was elongated in HEL cells during LY2784544 incubation. It would be of great interest to further determine how JAK2 inhibitors cause the elongation of telomere in MPN cells and how the treatment failure occurs.

We found that telomerase inhibitor GRN163L decreased the accumulation of CD34+ HEL cells mediated by LY2784544 treatment. Also, the elongation of telomere length caused by LY2784544 could be reversed by GRN163L treatment. The accumulation of CD34+ cells was also found to be present in CD34- cells, suggesting the treatment of LY2784544 might convert cells to a more immature status. However, the remaining CD34+ cells after CD34+ cell isolation that were resistant to the LY2784544 treatment may also result in the accumulation of CD34+ cells in CD34- cells. If so, our study showed that the CD34+ HEL cells could tolerate LY2784544 more than CD34- cells.

In summary, we here demonstrated that JAK2 inhibitor LY2784544 caused the enrichment of CD34+ cells through induction of KLF4 expression in the $JAK2^{V617F}$ HEL cell line. This phenomenon was attenuated by KLF4 silencing. Targeting both KLF4 and $JAK2^{V617F}$ may have better efficacy for the treatment of MPNs. The telomerase inhibitor GRN163L could reverse the accumulation of CD34+ cells after LY2784544 treatment, which suggests the potential therapeutic effect of combination of JAK2 inhibitors and telomerase inhibitors in the treatment of MPNs.

6.3 INTRINSIC 5-LIPOXYGENASE ACTIVITY REGULATES THE MIGRATION AND ADHESION OF MANTLE B-CELL LYMPHOMA CELLS

The migration and adhesion of MCL cells is a very important mechanism for the spread of these cells in the body. Thus, the inhibition of cellular migration and adhesion will reduce dissemination to secondary lymphatic tissues via systemic circulation. Here, we found that the inhibition of the 5-LOX pathway through 5-LOX or FLAP inhibitors could result in the decrease of the migration and adherence ability of the MCL cell line JeKo-1 in vitro, and this inhibitory effect was as potent as BTK inhibitor Ibrutinib. It was reported that the main mechanism of ibrutinib affecting tumors was to change the redistribution status of the malignant cells rather than cytotoxicity (108).

We also found that the effect of 5-LOX/FLAP inhibitors on cell migration was dependent on the 5-LOX expression since these inhibitors did not inhibit the migration of the T cell lines Jurkat and Molt-3, which are 5-LOX negative (92). Furthermore, CRISPR/Cas9 mediated deletion of the *ALOX5* gene in JeKo-1 cells caused the decrease in the migration of JeKo-1 cells.

It has been known that most of the B lymphocytes contain high amounts of 5-LOX protein, but germinal center B cells express very low or no amounts of 5-LOX protein (91, 109, 110). Likely, the main role of the 5-LOX pathway in B cells is to guide the cells to secondary lymphoid organs where the cells are either transformed into plasma cells or undergo apoptosis. Cells trafficking to secondary lymphatic tissues is a way to escape from the immune system and thereby reduce cell apoptosis in malignant cells (111, 112). Secondary lymphatic stromal cells are known to generate chemoattract CXCL12 and some other chemokines, which guide malignant B cells to move towards lymph node compartments (113). The chemokine CXCL12 induces cell chemotaxis and adhesion. CXCL12 is playing a role through binding to its receptor CXCR4. The crosstalk between malignant B-cells and the stromal micro-environment is an essential mechanism in the spread of the MCL cells (114). Our results showed that the adherence ability of JeKo-1 cells to stromal cells was inhibited by 5-LOX/FLAP inhibitors.

Interestingly, our study showed that LTB_4 did not induce the migration of B cells, but it is well-known that LTB_4 is a potent chemotactic agent for neutrophils and T cells (76). LTB_4 hasn´t been reported to be released through the activation with calcium ionophore or under physiological conditions in normal or malignant B lymphocytes (115). Moreover, LTB_4 was not chemotactic for MCL cells. These results suggest an additional intracellular signaling role for 5-LOX derived mediators in B lymphocytes that is different from their well-known function as metabolites promptly released from the cells upon activation. This indicates that 5-LOX protein in B lymphocytes may play an intrinsic role in cells. Figure 14 is a drawing showing a proposed effect of 5-LOX/FLAP inhibitors. Previous studies have shown an intrinsic function of the 5-LOX pathway in acute myeloid leukemia cells (116) and neutrophils (117, 118). Moreover, the intrinsic neutrophil 5-LOX activity regulates the adherence and chemotaxis of neutrophils in vitro (117).

In fact, there are also several other studies demonstrating that 5-LOX has an intrinsic function. It has been shown that 5-LOX is related to the pathophysiological function through the interplay with various nuclear proteins such as dicer, p53, and the Wnt/beta-catenin signaling pathway (119). The modulation of microRNA by 5-LOX is another important non-canonical function of this enzyme (119, 120). According to previous studies, 5-LOX was found mostly

located in the nucleus of tissue B lymphocytes (115) and the production of LTs could also be found in the nucleus (121, 122). Thus, we could not rule out the possibility that certain biological actions of 5-LOX might be due to its ability to generate the reactive oxygen species or LTs in the cell nucleus.

The phosphorylation of 5-LOX through different kinds of kinases has also been shown to activate the enzyme or be related to its transportation between cytoplasm and cell nucleus (123). A partial of 5-LOX is very likely to be phosphorylated continuously at Ser 523 in B-cells (124). It remains to be further explored if this phosphorylation of 5-LOX is correlated with the change from an extrinsic role to its intrinsic function of the enzyme in B lymphocytes. Taken together, it is likely that the intrinsic function of 5-LOX in B cells play an important biological role in these cells.

By now, two different FLAP inhibitors have entered clinical phase I and II studies for several diseases. GSK 2190915 has been used as the treatment strategy of asthma in a clinical phase II study (65). Another kind of FLAP inhibitor, AZD5718, has tentatively been shown to be useful for the treatment of coronary artery disease. So far, it has been tested in a phase I study. The LTB_4 levels in plasma decreased over 90 % and the drug was well-tolerated (125).

In summary, the present study demonstrates that 5-LOX/FLAP inhibitors inhibit migration and adherence of MCL cells, indicating that these compounds might be useful for the treatment of MCL. Clinical trials will show which MCL patient groups that may benefit from treatment with these drugs.

7 POINTS OF PERSPECTIVE

In paper I, we determined the DNA methylation status of the PBMCs from CD34+ cells and three B cell subpopulations from monozygotic triplets two of whom were diagnosed with HL, while the other remains HL free. We found the genes in several regions are hyper-methylated in Naïve-B cells and Marginal zone-like B-cells in both HL triplets compared with the HL free triplet. Some of these genes are related to cancer development or hematopoiesis. It helps us to understand the pathogenesis of HL. Further study, such as other epigenetic changes that happened in other twin studies in HL and other hematological diseases, could further help us to understand the development of hematopoiesis, and the role of epigenetics in the pathogenesis of HL.

In paper II, we found that JAK2 inhibitor LY2784544 caused the accumulation of CD34+ cells in HEL cell line which carries $JAK2^{V617F}$ mutation, coupled with KLF4 upregulation. The combination of KLF4 and JAK2 inhibitions or JAK2 plus telomerase inhibitors may be potentially used as a therapeutic strategy for MPNs. In future studies, how JAK2 inhibition causes the KLF4 upregulation is needed to be further clarified.

In paper III, we demonstrated that 5-LOX/FLAP inhibitors inhibit migration and adherence of MCL cells, indicating that these compounds might be useful for the treatment and MCL. We will continue with the study of the mechanism of how 5-LOX pathway influences the migration of B-cells, and importantly, other clinical trials also need to show which MCL patient groups may benefit from treatment with these drugs.

8 ACKNOWLEDGEMENTS

After a long journey, finally, it comes to my big day, there are so many thanks I would like to express now.

I would like to say thanks to my main supervisor, **Dawei Xu**, thank you for your kind help with my research and my life here. I still remember the first day when I came to the lab, you invited me for lunch at CMM. Since then, I have been experiencing your strict attitude with scientific research, you are one of the most hard-working scientists I have ever met. You are a good model for us all.

My co-supervisor, **Magnus Björkholm**, thanks a lot for your excellent supervision. You are so good at communicating with people from all research areas. You are always so gentle and polite, make everyone feel very comfortable. You have been leading our group for so long, thanks a lot for all the lovely Christmas gifts.

My co-supervisor, **Hans-Erik Claesson**, you provide me so many advices on my projects, thanks for being so patient with me, you are always so helpful and kind, thank you for sharing your traveling experience and always being so supportive. I will always remember your funny magics.

My co-supervisor, **Jan Sjöberg**, thanks for all your kindness and understanding of me. I still remember the moment that we talked about your daughter when she had a baby, you were so happy. I went to Uppsala to experience the city you have been working for a long time, it is a beautiful city.

My co-supervisor, **Cheng Liu**, thanks a lot for introducing me to scientific research, you taught me so many useful lab techniques. Thanks for your endless encouragement and trust, I hope all your families be healthy and happy.

My supervisor at Shandong university, **Yidong Fan**, thanks so much for your help during my study in China and Sweden, you are always so kind, so gentle to everyone, you are a great leader and always inspire me in my life.

My mentor, **Mingmei Shang**, you did help me a lot to get used to the life and study here. I still remember that you taught me how to run WB, PCR and cell culture, thanks for being my mentor.

Klas Strååt, thanks for being our lab manager, you offered me so much help and support as wells as advices on my projects. Hope everything goes on well with you and your families.

Sahra Bunner and **Maritta thorén,** thanks for your help with our admin work. **Lillemor Melander,** thanks so much for PhD education work. **Anne Sundblad,** for all the meetings we have together.

All my **co-authors**, **Thale Kristin Olsen, Tomas J Ekström, A. Ali Zirakzadeh, Radwa Almamoun, Louise K Sjöholm, John Inge Johnsen, Ola Winqvist, Laia Sadeghi, Magali Merrien, Anthony P. Wright, Birgitta Sander,** thanks a lot for your professional advice with my projects and manuscripts.

Jenny Dahlström, thanks for being patient and kind even when you were so busy with your work. **Xiaotian Yuan** and **Lizhou Fan**, thanks for your kind care and delicious treat, the moment we watched the Barcelona football game together provided me so much happiness and excitement. **Jingya Yu**, thanks for your help during my study, you are always the smartest girl. **Yujiao Wu**, you are a model for me, both in the life and research field. **Xiangling Xing**, thanks for all the time we spent together in the lab. **Zhiqing Fang** and **Ning Zhang**, thanks for being so kind and provide us so much happiness and joy. **Liu Yang**, thank you for being so patient and kind to me. **Linghua Kong** and **Mailin Zhou**, thanks for all the delicious meals, all the happy time we spent together, I will never forget the time we went boating, camping, fishing, and walking together.

My friends and colleagues, **Yanxia, Liyuan, Jingjing, Bingnan, Xiaolu, Xiuming**, **Hongya, Qingyang Xiao**, **Jiwei Gao, Ziqing Chen**, **Yaxuan, Kia, Tom, Mikael, JaKob, Annika, Katarina, Malin, Jinming Han, Hao Shi, Xia Hao, Min Guo**, **Muyi Yang, Chenfei He, Na Wang, Dang Wei**, **Konstantina, Huaitao, Nini Mu**, **Yuanyuan**, **Shuai Tan**, **Zhengye**, **Georgios, Xue Zhang, Li Wei**, **Jikai, Luchao, Xiaofeng, Zhengfang, Li Yang, Kun Wang, Keqiang, Min Xu, Yicai Yue**, **Yan**, **Xinxia**, **Shuo Xu**, **MingKai ,** thanks for all your company and support.

I would like to thank my families, **my father**, **my mother**, **my brother** and **my sister**, you are the source of my happiness, thank you for your support.

This study was supported by Cancer Society in Stockholm, Swedish Cancer Society, Stockholm County Council and the Swedish Research Council, Knut and Alice Wallenberg Foundation, I would also like to thank China Scholarship Council for the scholarship.

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